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Saturated Fatty Acid and TLR Signaling Link β Cell Dysfunction and Islet Inflammation

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SUMMARY

Consumption of foods high in saturated fatty acids (FAs) as well as elevated levels of circulating free FAs are known to be associated with T2D. Though previous studies showed inflammation is crucially involved in the development of insulin resistance, how inflammation contributes to β cell dysfunction has remained unclear. We report here the saturated FA palmitate induces β cell dysfunction in vivo by activating inflammatory processes within islets. Through a combination of in vivo and in vitro studies, we show β cells respond to palmitate via the TLR4/MyD88 pathway and produce chemokines that recruit CD11b^{+}Ly-6C^{+} M1-type proinflammatory monocytes/macrophages to the islets. Depletion of M1-type cells protected mice from palmitate-induced β cell dysfunction. Islet inflammation also plays an essential role in β cell dysfunction in T2D mouse models. Collectively, these results demonstrate a clear mechanistic link between β cell dysfunction and inflammation mediated at least in part via the FFA-TLR4/MyD88 pathway.

INTRODUCTION

Obesity has reached epidemic proportions around the world due to a modern lifestyle characterized by increased consumption of foods rich in energy and saturated fat, combined with reduced physical activity (Hill, 2006). This obesity epidemic has resulted in dramatic increases in the incidences of obesity-associated diseases, including type 2 diabetes (T2D), and represents a worldwide health crisis. Numerous studies have shown that insulin resistance precedes the development of hyperglycemia in subjects that eventually develop T2D (Prentki and Nolan, 2006). However, T2D only develops in insulin-resistant subjects after the onset of pancreatic β cell dysfunction (Leahy, 2005).

Inflammation is increasingly known to play a key role in the initiation and development of obesity-associated diseases. For instance, elevated levels of circulating proinflammatory cytokines are observed in obese subjects and those with T2D (Schenk et al., 2008). Mechanistically, proinflammatory signaling pathways can inhibit insulin signaling, providing a link between inflammation and insulin resistance. By contrast, the involvement of inflammation in β cell failure in T2D is poorly understood, although a T cell-mediated autoimmune response against β cells appears to be a primary pathogenic mechanism in type 1 diabetes (T1D) (Mallone and van Endert, 2008). Recent reports suggest similar inflammatory processes are also activated within T2D islets. For instance, expression of interleukin (IL)-1β, which is involved in the autoimmune processes leading to T1D, is upregulated in the islets of patients and animal models with T2D, and an IL-1 receptor antagonist reportedly improves blood glucose levels and β cell function in T2D patients (Donath and Shoelson, 2011; Larsen et al., 2007). Accumulation of macrophages within islets has also been observed in T2D subjects (Richardson et al., 2009), further suggesting activation of inflammation. However, the mechanism by which the accumulation of macrophages within islets is initiated and the extent to which the accumulated macrophages contribute to β cell dysfunction is not yet clear.

Free fatty acid (FFA) levels are elevated in obese subjects (Boden, 2008) and are an independent predictor of future T2D (Charles et al., 1997). High intake of saturated fatty acids (FAs) has also been linked to higher risks of T2D (Risérus et al., 2009) and coronary artery disease. Previous studies have shown that palmitate, the most abundant saturated FFA in blood, inhibits insulin signaling in liver, muscle, and fat cells in vitro

(Dey et al., 2007; Hommelberg et al., 2009; Nakamura et al., 2009). These studies have also shown the deleterious effects of FFAs, collectively termed "lipotoxicity," on β cells in vitro (Weinberg, 2006). Based mainly on the findings of in vitro studies, it was proposed that β cell lipotoxicity is directly induced by palmitate at least in part via pathways involving endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) (Cnop, 2008; Fonseca et al., 2011). Similarly, in vivo infusion of emulsified soybean triglyceride impairs β cell function in rats and humans (van Raalte et al., 2010). However, the molecular mechanisms by which FFAs induce β cell dysfunction in vivo remain poorly understood.

Potential molecular links between FFAs and metabolic disease are the toll-like receptors (TLRs). TLRs are pattern recognition receptors that initiate innate immune responses upon recognition of a wide range of pathogen-associated molecular patterns, including lipids, lipoproteins, and proteins (Kawai and Akira, 2009). A growing body of evidence suggests they are also capable of responding to endogenous ligands, including lipids (Fessler et al., 2009). For instance, saturated FAs activate the inflammatory program via TLR4 in macrophages (Lee et al., 2001; Suganami and Ogawa, 2010). Moreover, recent studies showed that TLR4 signaling is important for high fat diet-induced insulin resistance, presumably by mediating inflammatory responses within adipose tissue and skeletal muscle (Shi et al., 2006; Suganami and Ogawa, 2010). Within islets, TLR4 is expressed in β cells (Goldberg et al., 2007), and the chemokine CXCL10 was recently reported to bind to TLR4 and induce β cell apoptosis (Schulthess et al., 2009). Collectively, these studies suggest that the TLR4 pathway may play a key role in β cell dysfunction in T2D.

In the present study, we established a method to selectively increase the circulating free palmitate level in mice and analyzed its effects on β cell function. We found that palmitate induces β cell dysfunction in vivo by activating inflammatory processes within islets. We also show that β cells sense palmitate via the TLR4/MyD88 pathway and produce chemokines, which in turn recruit M1-type proinflammatory monocytes/macrophages to the islets. Depletion of M1-type cells ameliorated the β cell dysfunction induced by palmitate, confirming the causative involvement of M1-type cells. M1 macrophage accumulation was also important for β cell dysfunction in T2D models db/db and KKAy mice. Taken together, the results of the present study establish a molecular link between islet inflammation and β cell dysfunction in T2D.

RESULTS

Palmitate Induces β Cell Dysfunction In Vivo

Saturated FAs such as palmitate reportedly exert adverse effects on a variety of cultured cells in vitro, including β cells (Cnop, 2008; Prentki and Nolan, 2006). To the best of our knowledge, however, the effects of palmitate on β cells have never been directly tested in vivo by selectively increasing circulating palmitate levels. Instead, infusion of lipids was previously accomplished using emulsified triglycerides derived from soybean or lard oil, which contain a variety of FA residues, often together with heparin, which activate lipoprotein lipase and promote FFA release from the infused triglycerides and from lipoproteins in the circulation (Teusink et al., 2003). Most likely, however, each FA species will affect cellular function differently. To eliminate the confounding effects of the multiple FA species, we initially established a method that enables us to selectively increase circulating palmitate levels. Because FA ethyl esters are known to be rapidly hydrolyzed in the blood of rodents (Hungund et al., 1995), we tested whether emulsified ethyl palmitate, which can be intravenously administered, could be used for that purpose. When emulsified ethyl palmitate was incubated in mouse serum, it was rapidly hydrolyzed to free (nonesterified) FA, as expected (Figure S1A). When the emulsified ethyl ester solution (600 mM) was continuously infused (0.2 μl/min) into mice via the jugular vein for 2 hr or 14 hr, the serum FFA levels were significantly increased (Figure S1B) and, more importantly, only the palmitate levels were significantly increased among the major FFA species, as compared with vehicle infusion (Figures 1A and S1C).

We then tested whether ethyl palmitate infusion would affect β cell function in mice. Intravenous glucose tolerance tests (IVGTTs) showed that the acute insulin response (AIR) (Marcelli-Tourvieille et al., 2006) was increased by a 14 hr infusion of ethyl palmitate, as compared to the baseline level, while longer infusions progressively impaired acute insulin secretion (Figures S1D and S1E). These results are compatible with earlier findings that, in both mice and humans, a short term infusion of emulsified soybean triglycerides plus heparin augmented glucose-induced insulin secretion (GSIS), in part via the GPR40 FFA receptor (Steneberg et al., 2005), while longer infusion impaired it (Carpentier et al., 2000). Importantly, however, the expression levels of genes involved in β cell function, including *Pdx1*, *Insulin* (mature *Ins1* and *Ins2* mRNA), and *Ins2* pre-mRNA (which more accurately reflects transcription rates) (Iype et al., 2005), were already significantly reduced in islets from mice infused with ethyl palmitate for 14 hr, as compared to those from mice infused with vehicle (Figure 1B). These results strongly suggest that continuous ethyl palmitate infusion induces β cell dysfunction within 14 hr. Indeed, GSIS in islets isolated from mice infused with ethyl palmitate was significantly diminished, as compared to the vehicle-infused controls (Figure 1C). And when increasing doses of ethyl palmitate (0.72, 2.2, 7.2 μmol/hr) were infused, there tended to be a dose dependency in the suppression of gene expression and the levels of PDX-1 protein (Figures S1G and S1H). It thus appears that palmitate rapidly induces β cell dysfunction in vivo, despite the apparent early augmentation of insulin secretion. We therefore focused on the mechanism by which palmitate induces rapid β cell dysfunction.

The TLR4/MyD88 Pathway Is Essential for Palmitate-Induced β Cell Dysfunction In Vivo, but Not In Vitro

TLR4 is known to mediate the response to palmitate in macrophages in vitro (Suganami and Ogawa, 2010). TLR4 is expressed in β cells (Goldberg et al., 2007; Vives-Pi et al., 2003), and the binding of chemokine CXCL10 to TLR4 induces β cell apoptosis (Schulthess et al., 2009). These earlier findings prompted us to test whether TLR4 signaling might be involved in palmitate-induced β cell dysfunction. When we infused ethyl palmitate into *Tlr4* and *Myd88* (encoding TLR4's adaptor protein MyD88) knockout mice, the infusion did not reduce expression of *Pdx1*, *Insulin*, and *Ins2* pre-mRNA or PDX1 protein in the islets of

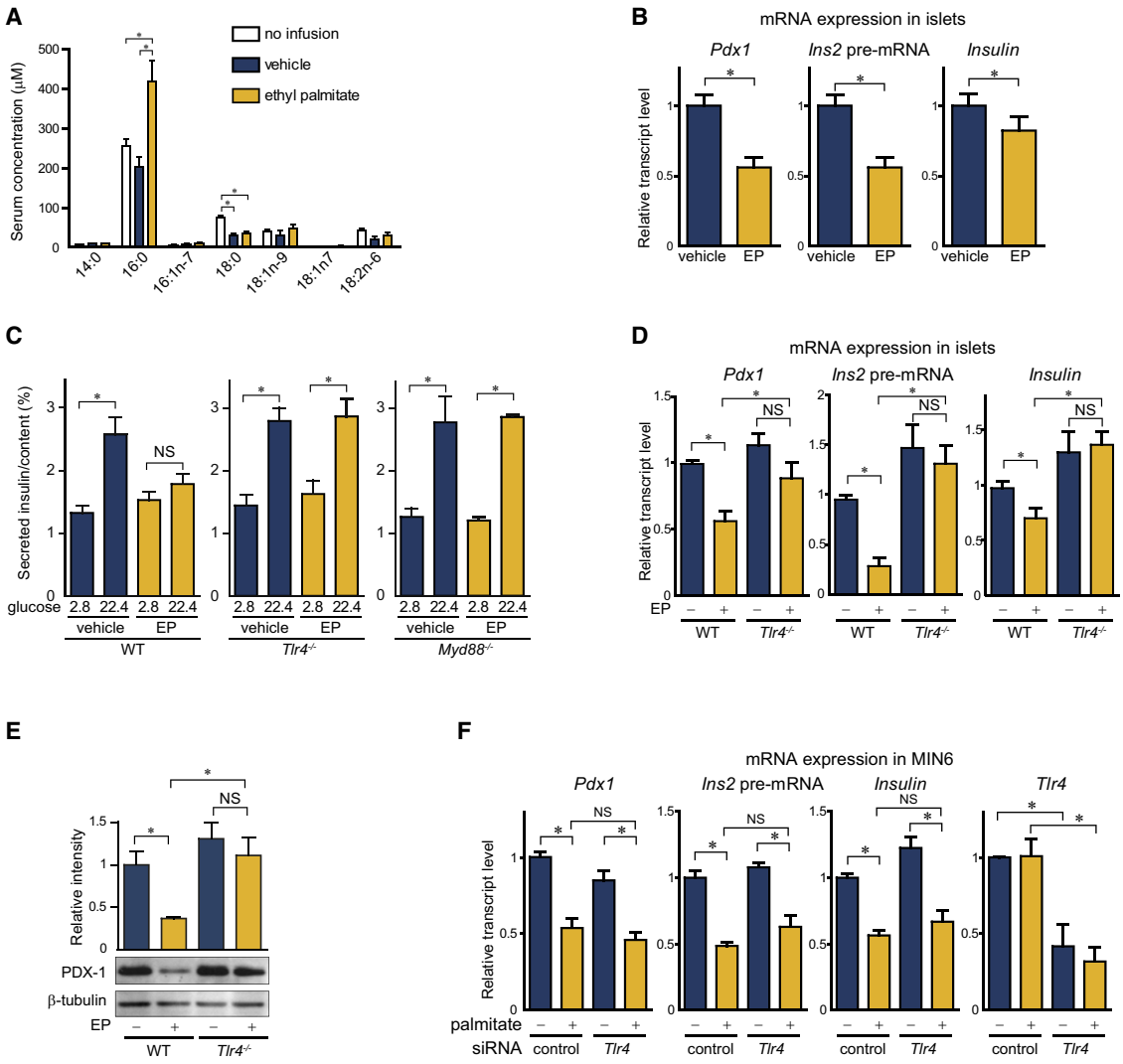

Figure 1. Ethyl Palmitate Infusion Induces β Cell Dysfunction via the TLR4/MyD88 Pathway

(A) Mice were continuously infused for 14 hr with ethyl palmitate or vehicle, after which serum levels of each free FA were analyzed. n = 4 mice for each group. *p < 0.05. Error bars, SEM.

(B) Effects of ethyl palmitate infusion on expression of β cell-associated genes. Islets were collected from mice infused for 14 hr with ethyl palmitate or vehicle. Levels of mRNA expression were analyzed using real-time PCR. mRNA expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in vehicle-treated mice. n = 4 mice in each group.

(C) Effects of ethyl palmitate infusion on GSIS. The islets isolated from wild-type (WT), *Tlr4*$^{-/-}$, and *Myd88*$^{-/-}$ mice infused for 14 hr with ethyl palmitate or vehicle were stimulated for 1 hr with KRB buffer containing 2.8 mM or 22.4 mM glucose, and the insulin secreted into the buffer was assayed. Insulin levels in the buffer were normalized to the insulin content. n = 5–9 islet preparations. Shown are representative data from three or more experiments.

(D and E) Wild-type and *Tlr4*$^{-/-}$ mice were infused for 14 hr with ethyl palmitate or vehicle, after which expression of β cell-associated genes (D) and PDX-1 protein (E) was analyzed in islets isolated from the indicated mice. Expression levels were normalized to the levels in vehicle-treated wild-type mice. For the western analysis, a representative blot image and relative band intensities are shown. Band intensities were normalized first to those of β-tubulin and then further normalized to the intensities in vehicle-treated mice. In (D), n = 6 mice in each group; in (E), n = 6 and 5 mice for wild-type and *Tlr4*$^{-/-}$, respectively.

(F) MIN6 β cells were transfected with control siRNA or siRNA targeting *Tlr4* and treated with 300 μM palmitate for 48 hr. Levels of mRNA expression of β cell-associated genes were analyzed. Expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in control cells. n = 4 in each group.

Tlr4$^{-/-}$ and *Myd88*$^{-/-}$ mice (Figures 1D, 1E, and S2A). Moreover, impairment of GSIS by ethyl palmitate was not observed in islets isolated from *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice (Figure 1C). Collectively, these data demonstrate that the TLR4/MyD88 pathway is essential for palmitate-induced β cell dysfunction.

We next used MIN6 β cells (Miyazaki et al., 1990) to further investigate the molecular mechanism by which palmitate reduces β cell-associated gene expression via the TLR4/MyD88 pathway. As expected, palmitate reduced expression levels of *Pdx1*, *Ins2* pre-mRNA, and *Insulin* (Figure 1F).

Unexpectedly, however, knocking down *Tlr4* and *Myd88* did not suppress the downregulation of those genes by palmitate (Figures 1F, S2C, and S2D), indicating the TLR4/MyD88 pathway was dispensable for palmitate-induced downregulation of β cell-associated genes in MIN6 cells. One possible explanation for the discrepancy between the in vivo and in vitro findings is the involvement of non-cell-autonomous mechanisms in the in vivo setting.

Palmitate Induces Accumulation of Proinflammatory Monocytes/Macrophages within Islets

To address the possible involvement of non-β cells in ethyl palmitate-induced β cell dysfunction, we analyzed the islet cells by flow cytometry and detected the presence of $CD11b^+$ cells within isolated islets. The cells were morphologically monocytes or macrophages, but not granulocytes (Figure 2A). Moreover, the majority of $CD11b^+$ cells were negative for Ly-6G, a granulocyte marker, but did not show the surface phenotypes of dendritic cells, indicating that islet $CD11b^+$ cells were mainly monocytes or macrophages (Figures S3A and S3B). To detect any contamination by circulating leukocytes, we also tested for the presence of red blood cells (RBCs) (Galkina et al., 2006). Based on the numbers of RBCs and leukocytes in blood, the fraction of contaminating blood leukocytes within islets was estimated to be < 0.0021% of the total live cells (see Figure S3C and Supplemental Experimental Procedures for further discussion). Thus, nearly all of the $CD11b^+$ cells appear to be located within the islets. Furthermore, whole-mount pancreas showed the presence of $CD11b^+$ cells within islets (Figures 2B and S3D), and F4/80 staining of formalin-fixed pancreatic sections showed the presence of $F4/80^+$ macrophages (Figure 2C).

In the following flow cytometric analyses of $CD11b^+$ cells, we analyzed the cells gated to R1 (Figure S3E), because the majority of $CD11b^+$ cells were located there (Figure S3F).

Ethyl palmitate infusion increased the size of the overall $CD11b^+$ cell fraction (Figure 2D). Because recent studies have shown that macrophages can be divided into several subtypes (Mosser and Edwards, 2008), we further characterized the surface phenotypes of $CD11b^+$ cells. We found that ethyl palmitate infusion significantly increased the relative cell fraction of viable $CD11b^+Ly\text{-}6C^+$ cells (cells in region R2 in Figure 2E), whereas the $CD11b^+Ly\text{-}6C^-$ (R3) cell fraction was not significantly affected (Figure 2E). Similarly, when the numbers of $CD11b^+$ cells were expressed as total cells per islet, only $CD11b^+Ly\text{-}6C^+$ cells were increased (Figure S4A).

F4/80 levels varied widely among $CD11b^+Ly\text{-}6C^+$ cells, from low to high (Figure S4B), suggesting the population contained both newly recruited monocytes and those in transition to becoming macrophages, as well as mature macrophages (Sunderkötter et al., 2004; Swirski et al., 2007). $CD11b^+Ly\text{-}6C^-$ cells were largely positive for F4/80 (Figure S4B). Hereafter, these cell types will be designated as $CD11b^+Ly\text{-}6C^+$ monocytes/macrophages and $CD11b^+Ly\text{-}6C^-$ macrophages.

To further characterize the $CD11b^+$ cells, we first sorted them from the islets (Figure S4C). We then found that $CD11b^+Ly\text{-}6C^+$ cells expressed *Il1b* (encoding IL-1β) and *Tnfa* (encoding TNF-α), both of which induce β cell dysfunction (Bendtzen et al., 1986), more strongly than $CD11b^+Ly\text{-}6C^-$ macrophages (Figure 2F). By contrast, $CD11b^+Ly\text{-}6C^-$ macrophages expressed *Il10* (Figure 2F). In addition, surface expression of the M2 markers CD206 and CD301 was significantly higher in $CD11b^+Ly\text{-}6C^-$ cells than $CD11b^+Ly\text{-}6C^+$ cells (Figure S4D). Thus $CD11b^+Ly\text{-}6C^+$ cells exhibit the phenotype of M1 classically activated inflammatory macrophages, while the characteristics of $CD11b^+Ly\text{-}6C^-$ macrophages suggest an alternatively activated M2-like phenotype (Mosser and Edwards, 2008).

Ethyl palmitate infusion did not alter the $CD11b^+Ly\text{-}6C^+$ macrophage populations in the exocrine pancreas (Figure S4E) or the levels of total and $Ly\text{-}6C^+$ inflammatory monocytes in the circulation (Figure S4F). Taken together, these findings indicate that palmitate selectively promotes accumulation of $CD11b^+Ly\text{-}6C^+$ M1-type monocytes/macrophages within pancreatic islets.

Islet Cells Are Responsible for Palmitate-Induced Inflammatory Macrophage Accumulation

We next addressed the mechanism by which palmitate induces accumulation of inflammatory macrophages within islets. We found that ethyl palmitate infusion did not induce accumulation of $CD11b^+Ly\text{-}6C^+$ cells in the islets of $Tlr4^{-/-}$ and $Myd88^{-/-}$ mice, indicating that the TLR4/MyD88 pathway is involved (Figures 3A and S5A).

We then asked which cell types are responsible for initiating $CD11b^+Ly\text{-}6C^+$ cell accumulation. Because the TLR4/MyD88 pathway is known to be important for immune cell function (Kawai and Akira, 2009), we tested whether the absence of TLR4/MyD88 signaling in bone marrow (BM)-derived cells might underlie the reduced macrophage accumulation in $Tlr4^{-/-}$ and $Myd88^{-/-}$ mice. We generated *Tlr4* knockout mice whose BM was replaced with wild-type BM. Flow cytometric analysis using Ly-5.1 antibody indicated that > 85% of peripheral blood leukocytes were reconstituted in the BM-transplant recipient mice (data not shown). In the wild-type mice with wild-type BM, palmitate induced accumulation of $CD11b^+Ly\text{-}6C^+$ cells within the islets (Figure 3B). In $Tlr4^{-/-}$ mice with the wild-type BM, however, ethyl palmitate infusion failed to increase the $CD11b^+Ly\text{-}6C^+$ cell fraction within the islets, indicating that restoration of the TLR4 pathway in BM-derived cells was not sufficient to recruit $CD11b^+Ly\text{-}6C^+$ cells to the islets.

We also generated mice whose BM was replaced with $Tlr4^{-/-}$ BM. Interestingly, in response to ethyl palmitate, $Tlr4^{-/-}$ $CD11b^+Ly\text{-}6C^+$ cells accumulated within islets as efficiently as wild-type cells did (Figure 3C). Clearly, while a TLR4 pathway in non-BM cells is indispensable for palmitate-induced recruitment of M1-type cells to islets, TLR4 appears to be dispensable for the initial accumulation of M1-type cells. BM *Tlr4* deletion also did not significantly reduce the expression of *Il1b* or *Tnfa* within islets in response to ethyl palmitate, though *Il1b* levels tended to be modestly reduced (Figure S5B). These results suggest that *Tlr4* in hematopoietic cells is dispensable, at least for the initial activation of inflammatory processes.

β Cells Initiate Macrophage Accumulation by Responding to Palmitate via the TLR4/MyD88 Pathway

We hypothesized that β cells initiate macrophage recruitment in response to palmitate. To test that idea, we first assessed the capacity of β cells to promote macrophage recruitment using cultured cells. We found that medium conditioned by

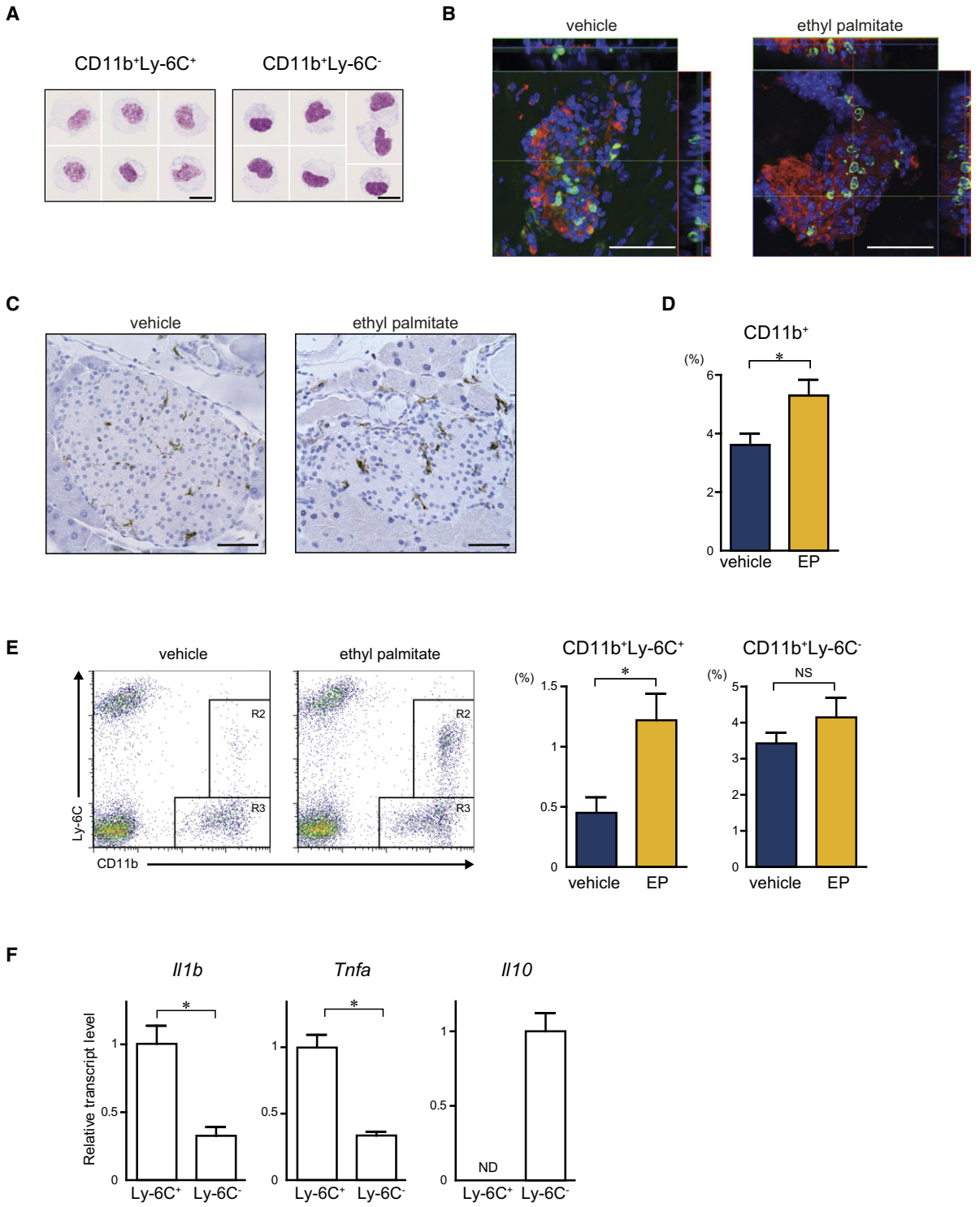

Figure 2. Palmitate Induces Accumulation of Proinflammatory Macrophages within Islets

(A) Representative cytospin preparations of CD11b$^+$Ly-6C$^+$ and CD11b$^+$Ly-6C$^-$ cells isolated from mice infused with ethyl palmitate. Cells were stained with Giemsa. Scale bar, 10 μm.

(B and C) Immunohistochemical identification of CD11b$^+$ (B) and F4/80$^+$ (C) cells in islets. For CD11b staining, whole-mount pancreatic tissues from mice infused for 14 hr with vehicle or ethyl palmitate (EP) were immunostained for CD11b and insulin. Representative planes from the Z-stack images are shown. The maximum intensity projection views are shown in Figure S3D. Nuclei were counterstained with Hoechst 33342. Scale bars, 50 μm. CD11b, green; insulin, red; nuclei, blue. For F4/80 staining, formalin-fixed paraffin-embedded sections of pancreas were immunostained for F4/80 (brown). Nuclei were counterstained with hematoxylin. Scale bar, 50 μm.

palmitate-treated MIN6 β cells induced migration of RAW264.7 cells more effectively than medium conditioned by vehicle-treated cells, and knocking down *Tlr4* and *Myd88* in MIN6 cells using specific siRNAs abolished the induction of RAW264.7 migration by conditioned medium (Figure 3D).

We next addressed the mechanisms by which β cells promote macrophage accumulation in islets. We systematically analyzed secretion of chemokines and cytokines from MIN6 cells (Figure S5C). We found that palmitate induced expression and secretion of the chemokines CCL2/MCP-1 and CXCL1/chemokine KC in MIN6 cells, and that knocking down *Tlr4* and *Myd88* suppressed the palmitate-induced upregulation of *Ccl2* and *Cxcl1* and their secretion (Figures 3E, S5C, and S5D). Similarly, ethyl palmitate infusion induced chemokine expression within islets in vivo, and ablation of *Tlr4* and *Myd88* abolished that effect (Figures 3F and S2B). We then isolated β cells from islets and further confirmed that palmitate increases the cells' expression of *Ccl2* and *Cxcl1* in vivo (Figure 3G). To further test if β cells might respond to palmitate via TLR4, we cultured β cells isolated from islets of wild-type and *Tlr4*$^{-/-}$ mice. When isolated β cells were cultured in a dish coated with laminin, which was previously shown to support β cell survival and *Cxcl1* expression (Ribaux et al., 2007), the cells isolated from wild-type islets responded to palmitate by expressing *Cxcl1* (Figure S5E). By contrast, cells from *Tlr4*$^{-/-}$ islets did not respond to palmitate, indicating that β cells sense palmitate via TLR4. *Ccl2* expression was undetectable under these culture conditions. Taken together, these results indicate that β cells sense palmitate and express *Ccl2* and *Cxcl1* via TLR4/MyD88 signaling.

The NF-κB pathway is known to be activated by TLR4 and to control expression of chemokine genes (Kawai and Akira, 2009). As expected, palmitate rapidly (within 20 min) induced phosphorylation of IKK in MIN6 cells, and this effect was suppressed by knockdown of *Tlr4* (Figure 3H). Moreover, inhibitors of NF-κB signaling (BMS-345541 and SC-514) significantly suppressed the palmitate-induced upregulation of *Ccl2* and *Cxcl1* expression in MIN6 cells (Figure 3I). On native polyacrylamide gels, the band for the TLR4/MD2 complex was shifted in the presence of palmitate, as it was with lipid IVa, indicating that palmitate interacts with TLR4/MD2 (Figure S5F). Collectively then, these results indicate that palmitate induces expression of chemokine genes in β cells via TLR4/MyD88/NF-κB signaling.

Although ethyl palmitate was rapidly hydrolyzed to palmitate in the blood (Figures 1A and S1A–S1C), it was still possible that the unhydrolyzed ester might also stimulate chemokine expression via the TLR4/MyD88. To test this possibility we treated MIN6 cells with ethyl palmitate and found that, in contrast to palmitate, ethyl palmitate did not induce chemokine expression in MIN6 cells (Figure S5G). This supports the notion that palmitate, but not ethyl palmitate, is responsible for the observed effects of ethyl palmitate infusion on islets in vivo. High glucose levels might also affect chemokine expression as shown by the induction of IL-1β in isolated islets ex vivo (Maedler et al., 2002). However, infusion of ethyl palmitate either reduced or did not significantly affect blood glucose levels in wild-type, *Tlr4*$^{-/-}$, or *Myd88*$^{-/-}$ mice (Figure S5H). It is therefore very unlikely that glucose levels contribute to chemokine induction by ethyl palmitate in the present model.

We then tested whether CCL2 might be important for the accumulation of CD11b^{+}Ly-6C^{+} cells. We found that ethyl palmitate infusion was not capable of increasing CD11b^{+}Ly-6C^{+} populations in the islets of *Ccl2*$^{-/-}$ mice (Figure 3J), which indicates CCL2 is indispensable for the accumulation of these cells within islets in response to palmitate. Unexpectedly, however, the ethyl palmitate-induced CD11b^{+}Ly-6C^{+} cell accumulation was not affected by deletion of *Ccr2*, a gene encoding the CCL2 receptor (Figure S5I), which suggests a compensatory mechanism for CD11b^{+}Ly-6C^{+} cell recruitment is active in *Ccr2*$^{-/-}$ mice (Gutierrez et al., 2011).

The results presented so far strongly suggest that β cells sense palmitate via TLR4 and are responsible for the recruitment of inflammatory macrophages, in part via production of CCL2. However, it has been shown that endothelial cells and macrophages can also express *Ccl2*. We found that while *Ccl2* was expressed in CD11b^{-}Ly-6C^{+}CD31^{+} endothelial cells and resident CD11b^{+}Ly-6C^{-} macrophages, ethyl palmitate infusion did not alter its expression levels in these cell types (Figure S5J). Therefore, β cells appear to be the only population within islets that exhibits increased *Ccl2* expression in response to ethyl palmitate infusion. Although levels of *Ccl2* expression in β cells are lower than in resident macrophages, the β cell fraction in islets is much larger than the macrophage fraction. This makes it very likely that β cells are responsible for initiating the accumulation of CD11b^{+}Ly-6C^{+} cells in islets in response to palmitate. Once CD11b^{+}Ly-6C^{+} cells are recruited, they might also contribute to further recruitment.

Previous studies demonstrated that different FA species exert different effects on cellular function. We therefore compared effects of palmitate (C16:0), myristate (C14:0), and oleate (C18:1). Oleate modestly upregulated expression of *Ccl2* and *Cxcl1* in MIN6 cells, but myristate had no effect on expression of these genes (Figure 3K). We then continuously infused emulsified ethyl esters of the FAs and found that, whereas ethyl oleate stimulated the accumulation CD11b^{+}Ly-6C^{+} cells within islets, myristate did not affect the cell fraction (Figure 3L). These in vivo findings (Figure 3L) may reflect the effects on chemokine expression observed in vitro (Figure 3K).

Palmitate Induces Inflammatory Interactions between β Cells and Macrophages

Our results suggest that recruited inflammatory macrophages are involved in β cell dysfunction. Consistent with that notion

(D) Flow cytometric analysis of CD11b^{+} cells within the R1 region (Figure S3E) among cells isolated from mice infused for 14 hr with ethyl palmitate or vehicle. n = 6 mice for each group. *p < 0.05. Error bars, SEM.
(E) Flow cytometric analysis of the surface expression of CD11b and Ly-6C. Shown are representative plots and the CD11b^{+}Ly-6C^{+} and CD11b^{+}Ly-6C^{-} cell fractions among the total live cells. n = 3 mice for each group. The same data expressed as cell numbers per islet are shown in Figure S4A.
(F) mRNA expression of M1 and M2 marker genes in CD11b^{+}Ly-6C^{+} and CD11b^{+}Ly-6C^{-} cells within islets of mice administered ethyl palmitate. Expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in CD11b^{+}Ly-6C^{+} cells (for *Il1b* and *Tnfa*) or in CD11b^{+}Ly-6C^{-} cells (for *Il10*). n = 3 mice for each group. ND, nondetectable.

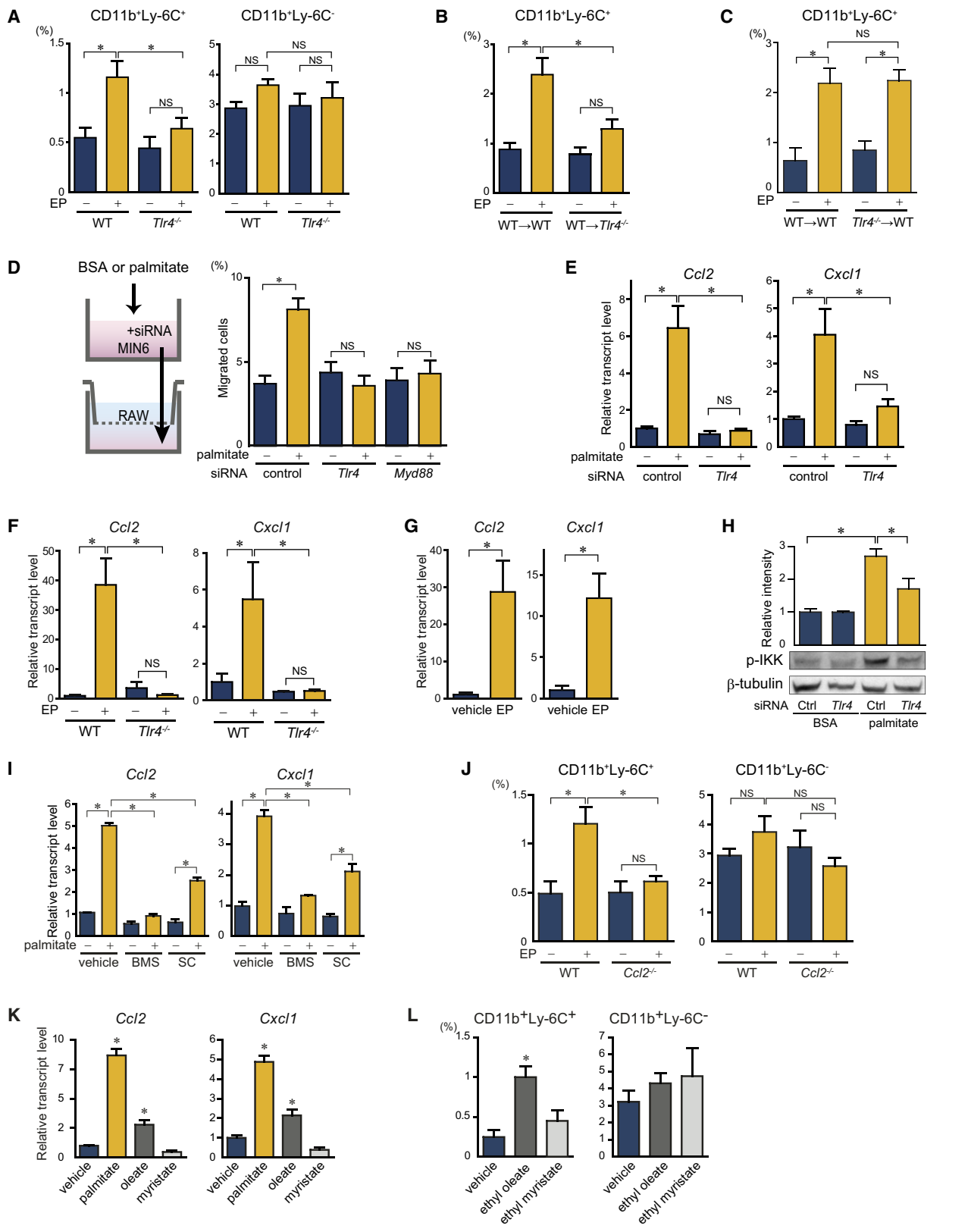

Figure 3. Palmitate Induces Chemokine Gene Expression in β Cells via the TLR4/MyD88 Pathway

(A) Wild-type and *Tlr4*$^{-/-}$ mice were infused for 14 hr with ethyl palmitate (EP) or vehicle, after which cells within islets were analyzed by flow cytometry. n = 4 mice for each group. *p < 0.05. Error bars, SEM.

and in contrast to the improved GSIS observed in MIN6 cells incubated for 14 hr in the presence of palmitate, coculturing MIN6 β cells with RAW264.7 cells (Figure 4A) or bone marrow-derived macrophages (Figure S6A) at a ratio of 100:1 significantly impaired GSIS in MIN6 cells. To test whether this apparent cellular interaction might be mediated by humoral factors, MIN6 and RAW264.7 cells were separately cultured in the bottom and inner wells of Boyden chambers, which permitted passage of humoral factors between the chamber wells. When RAW264.7 cells were present, palmitate reduced expression of *Pdx1*, *Ins2* pre-mRNA, and *Insulin*, as well as PDX1 protein, in MIN6 cells to a greater degree than when MIN6 cells were cultured alone (Figures 4B and 4C). Moreover, medium conditioned by palmitate-treated RAW264.7 cells reduced expression of β cell-associated genes in MIN6 cells, and a mixture of neutralizing antibodies against IL-1β and TNF-α partially suppressed the effects of the conditioned medium (Figure S6B). The conditioned medium also impaired GSIS in MIN6 cells, and the neutralizing antibodies partially restored GSIS (Figure S6C). Finally, addition of IL-1β and TNF-α to the culture medium suppressed expression of β cell-associated genes (Figure S6D). These results demonstrate that cytokines, including IL-1β and TNF-α, secreted from RAW264.7 cells impair MIN6 β cell function. The involvement of these cytokines in palmitate-induced β cell dysfunction in vivo is also supported by the finding that expression of *Il1b* and *Tnfa* within islets was increased by ethyl palmitate infusion and that this response was abolished in *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice (Figure S6E).

Interestingly, coculturing MIN6 with RAW264.7 cells markedly enhanced palmitate-induced expression of *Ccl2* and *Cxcl1* in MIN6 cells, as compared to MIN6 cells cultured alone (Figure 4B). In RAW264.7 cells, palmitate increased levels of *Il1b* and *Tnfa* expression (Figure 4D). Coculturing of RAW264.7 and MIN6 cells significantly augmented the palmitate-induced expression of *Il1b* and *Tnfa* in RAW264.7 cells, as compared to RAW264.7 cells cultured alone (Figure 4D). These results suggest that an interaction between RAW264.7 and MIN6 cells augments the inflammatory interactions. The findings that medium conditioned by palmitate-treated RAW264.7 cells induced *Ccl2* and *Cxcl1* in MIN6 cells and that this induction of chemokine genes was significantly inhibited by neutralizing antibodies against IL-1β and TNF-α (Figure S6B), indicate that the inflammatory interactions are at least partly mediated by cytokines. Thus, once CD11b^{+}Ly-6C^{+} cells had accumulated, the interactions between β cells and macrophages likely exacerbated β cell dysfunction and the inflammation induced by palmitate.

Inflammatory Macrophages Are Essential for Palmitate-Induced β Cell Dysfunction In Vivo

We next assessed the involvement of macrophages recruited to islets in palmitate-induced β cell dysfunction in vivo by depleting macrophages using clodronate-filled liposomes, which are ingested by monocytes and macrophages, after which intracellular release of clodronate causes apoptosis (Van Rooijen and Sanders, 1994). We intraperitoneally administered either clodronate or empty liposomes 54 hr and then 6 hr prior to the start of the ethyl palmitate infusion. As expected, the clodronate liposomes depleted macrophages in the liver and spleen, as well as circulating monocytes (data not shown), and they also inhibited the accumulation of CD11b^{+}Ly-6C^{+} inflammatory cells within islets in response to ethyl palmitate infusion (Figure 5A). They did not have a significant effect on the CD11b^{+}Ly-6C^{-} macrophage fractions, however. Because circulating Ly-6C^{+} inflammatory monocytes are known to be recruited to sites of inflammation, where they differentiate into M1 macrophages

(B) Wild-type and *Tlr4*$^{-/-}$ mice whose BM had been replaced with wild-type BM (WT → WT and WT → *Tlr4*$^{-/-}$) were infused for 14 hr with ethyl palmitate or vehicle, after which the CD11b^{+}Ly-6C^{+} cell fractions among islet cells were analyzed. WT BM → WT: vehicle n = 3 mice, ethyl palmitate n = 5; WT BM → *TLR4*$^{-/-}$: vehicle n = 3, ethyl palmitate n = 4.
(C) Wild-type mice whose BM had been replaced with either wild-type or *Tlr4*$^{-/-}$ BM (WT → WT and *Tlr4*$^{-/-}$ → WT) were infused for 14 hr with ethyl palmitate or vehicle, after which the CD11b^{+}Ly-6C^{+} cell fractions among the islet cells were analyzed. n = 3 and 4 mice for the vehicle and ethyl palmitate groups, respectively.
(D) Effect of MIN6-conditioned medium on RAW264.7 cell migration. MIN6 cells were transfected with control siRNA or siRNA targeting *Tlr4* or *Myd88* and treated with palmitate (300 μM) or vehicle, as indicated. Medium conditioned by MIN6 cells was added to the bottom wells of Boyden chambers, and the fractions of RAW264.7 cells that migrated through the porous membranes were determined. n = 4 wells for each group.
(E) MIN6 β cells were transfected with control siRNA or siRNA targeting *Tlr4* and treated with 300 μM palmitate for 48 hr as in Figure 1F. Levels of mRNA expression of chemokine genes were analyzed. Expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in control cells. n = 4 in each group. NS, not significant.
(F) Wild-type (WT) and *Tlr4*$^{-/-}$ mice were infused with ethyl palmitate or vehicle as in Figure 1B, and chemokine gene expression was analyzed in isolated islets. n = 5 and 6 mice for vehicle and ethyl palmitate, respectively.
(G) β cells were isolated from mice infused for 14 hr with ethyl palmitate or vehicle, and levels of mRNA expression were analyzed. n = 9 mice for each group.
(H) MIN6 β cells were transfected with either control siRNA or siRNA targeting *Tlr4* and then treated with 300 μM palmitate for 20 min. Phosphorylation of IKK was analyzed. Shown are representative blots and relative band intensities normalized to β-tubulin. n = 3.
(I) Effects of NF-κb inhibition on chemokine gene expression in MIN6 cells. The cells were incubated for 1 hr with either of the Iκb kinase inhibitors BMS-245541 (3 μM) or SC-514 (10 μM), or with vehicle. Thereafter, 300 μM palmitate or vehicle was added, and the incubation was continued for 14 hr. Gene expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in cells treated with vehicle and BSA. n = 3 in each group.
(J) Wild-type (WT) and *Ccl2*$^{-/-}$ mice were infused with vehicle or ethyl palmitate for 14 hr, after which the islet cells were analyzed by flow cytometry. n = 3 mice in each group.
(K) MIN6 cells were treated with 300 μM palmitate, myristate (C14:0), oleate (C18:1), or vehicle (BSA) for 14 hr, after which chemokine gene expression was analyzed. Expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in control cells. n = 4 in each group. *p < 0.05 versus vehicle. Error bars, SEM.
(L) Flow cytometric analysis of islet CD11b^{+} cells from mice administered various fatty acids. Mice were continuously infused for 14 hr with ethyl myristate, ethyl oleate, or vehicle, after which CD11b^{+} cells within islets isolated from these mice were analyzed by flow cytometry. n = 3 mice in each group. *p < 0.05 versus vehicle. Error bars, SEM.

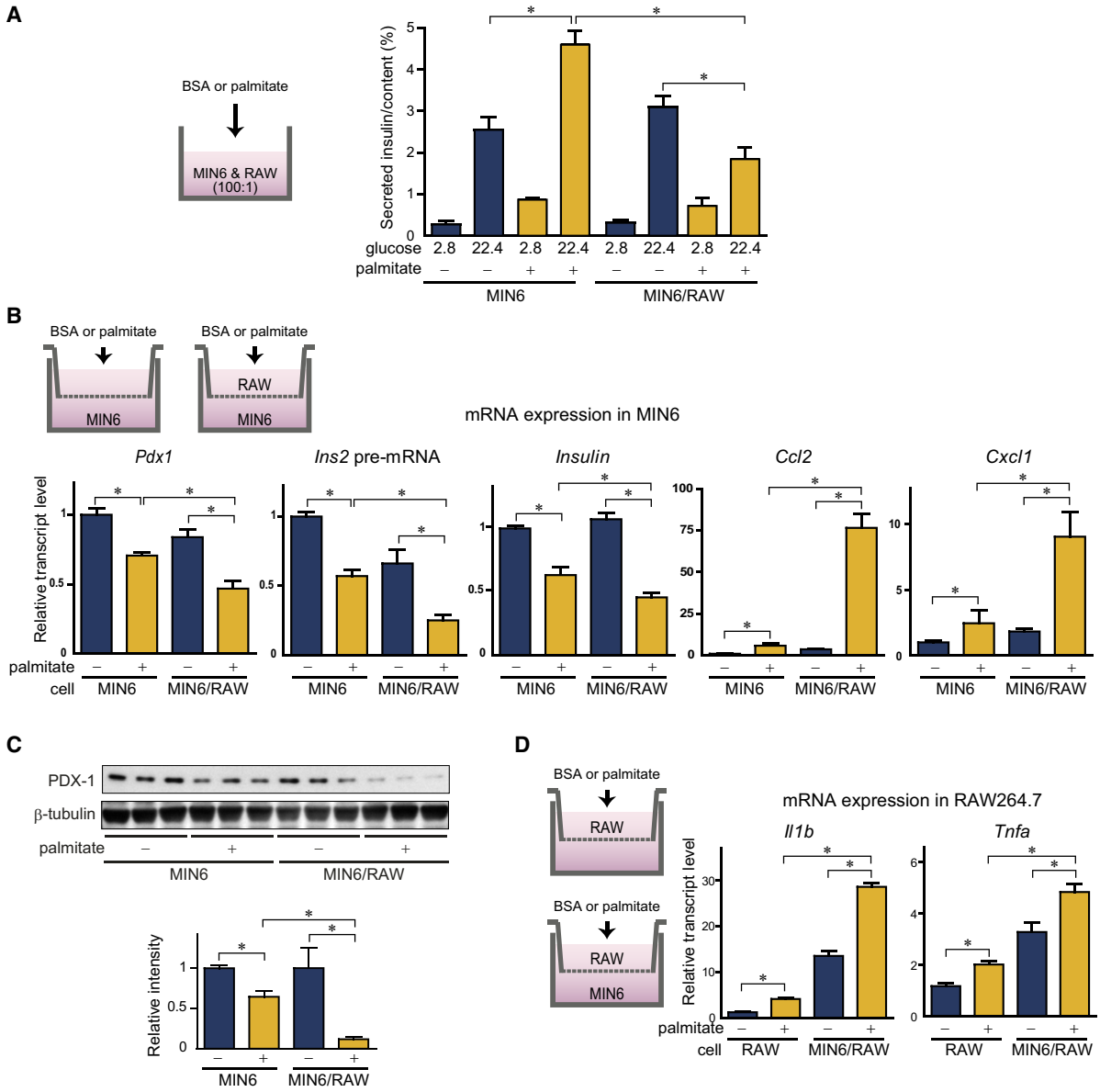

Figure 4. RAW264.7 Cells Induce MIN6 β Cell Dysfunction

(A) MIN6 cells were cocultured with RAW264.7 cells at a ratio of 100:1 (MIN6 versus RAW264.7) and then incubated for 14 hr with palmitate or vehicle, after which GSIS was analyzed. MIN6 cells cultured alone were similarly treated with palmitate and analyzed. n = 3 wells in each group. *$p < 0.05$. Error bars, SEM.

(B and C) MIN6 cells were plated in the bottom wells of Boyden chambers, with or without RAW264.7 cells in the inserts, as indicated. The cells were then treated for 48 hr with palmitate or vehicle, after which mRNA and PDX-1 protein expression levels in the MIN6 cells were analyzed. In (B) expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in MIN6 cells cultured alone and treated with vehicle. n = 5 wells in each group. In (C), levels of PDX-1 protein in MIN6 cells were analyzed by western blotting. Relative band intensities were normalized first to those of β-tubulin and then further normalized to the levels in MIN6 cells cultured alone and treated with vehicle. n = 3.

(D) RAW264.7 cells were plated in the inserts, with or without MIN6 cells in the bottom wells, and were treated with vehicle or palmitate as in (B). mRNA expression in RAW264.7 cells was then analyzed. Expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in RAW264.7 cells cultured alone and treated with vehicle. n = 3 in each group.

(Geissmann et al., 2010; Sunderkötter et al., 2004), these results strongly suggest that clodronate liposomes block the accumulation of $CD11b^+Ly\text{-}6C^+$ cells by depleting circulating monocytes, but they do not significantly affect resident $CD11b^+Ly\text{-}6C^-$ macrophages in the model we utilized.

Administration of clodronate liposomes abolished the palmitate-induced downregulation of *Pdx1*, *Insulin*, and *Ins2* pre-mRNA and inhibited the upregulation of *Il1b* and *Tnfa* within islets (Figure 5B). These in vivo findings, along with the results of the coculture experiments, demonstrate that $CD11b^+Ly\text{-}6C^+$

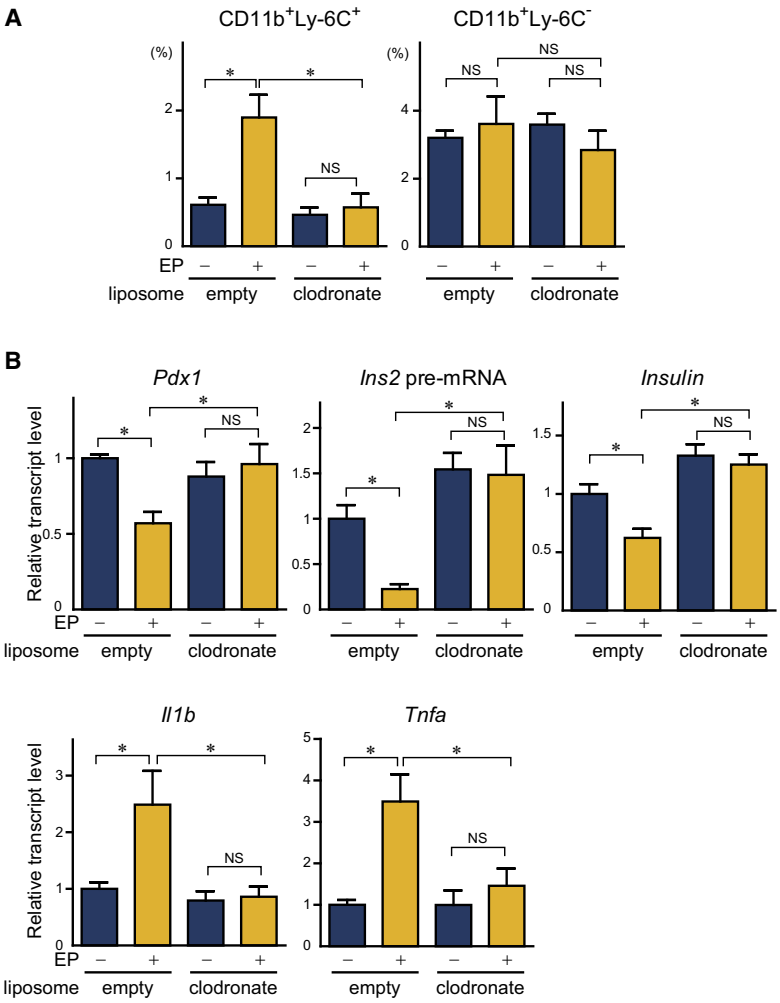

Figure 5. Recruited CD11b$^+$Ly-6C$^+$ Monocytes/Macrophages Induce β Cell Dysfunction

Clodronate or empty liposomes were intraperitoneally injected into mice 54 hr and then 6 hr prior to the start of ethyl palmitate infusion. The mice were then infused for 14 hr with ethyl palmitate or vehicle. In (A), islet macrophages were analyzed by flow cytometry. Vehicle: n = 3 mice; palmitate: n = 4. In (B), mRNA expression in islets was analyzed. Expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in mice administered empty liposomes and vehicle. n = 6 mice. *p < 0.05. Error bars, SEM.

inflammatory macrophages play a causative role in palmitate-induced β cell dysfunction.

Inflammatory Macrophages Play an Important Role in β Cell Dysfunction in db/db and KKAy Mice

Clinical studies have revealed an association between T2D and FFAs (Cnop, 2008; Risérus et al., 2009). Our results demonstrate that inflammatory processes involving M1-type monocytes/macrophages play an essential role in β cell dysfunction induced by palmitate and suggest that inflammation might be involved in the development of T2D. To begin testing this hypothesis, we analyzed the involvement of macrophages in β cell dysfunction in a well-established T2D model, db/db mice. We found that serum FFA levels were elevated in db/db mice, as compared to control db/+ mice (Figure S7A). We initially analyzed expression of PDX-1 and inflammatory cytokine genes within islets isolated from db/db and db/+ mice fed a normal chow diet. Levels of PDX-1 protein were reduced in the db/db pancreas (Figure 6A). On the other hand, levels of *Ccl2*, *Cxcl1*, *Il1b*, and *Tnfa* expression were significantly higher in db/db than db/+ islets (Figure 6B), indicating activation of inflammatory processes in the db/db islets.

Flow cytometric analysis showed that total islet CD11b$^+$ cell fractions did not significantly differ between db/+ and db/db mice (Figure 6C), though the CD11b$^+$Ly-6C$^+$ M1-type cell fraction was significantly larger in db/db than db/+ islets. By contrast, the CD11b$^+$Ly-6C$^-$ macrophage fractions were not significantly different between db/db and db/+ islets. To directly analyze the involvement of macrophages in the β cell dysfunction seen in db/db mice, we administrated clodronate liposomes to mice weekly, beginning when they were 5 weeks old. There were no significant differences in body weights between the two groups throughout the study (Figure S7B). Administration of clodronate liposomes suppressed the accumulation of CD11b$^+$Ly-6C$^+$ M1-type cells within islets (Figure 6D), while the CD11b$^+$Ly-6C$^-$ macrophage fractions tended to be reduced, though the difference was not significant. Levels of *Pdx1*, *Insulin*, and *Ins2* pre-mRNA expression were significantly lower in islets isolated from db/db mice administered empty liposomes than from untreated db/+ mice (Figure 6E). Clodronate liposome administration increased expression of *Insulin*, *Ins2* pre-mRNA, and *Pdx1* mRNA (Figure 6E). Although the upregulation of *Pdx1* mRNA expression was modest following clodronate liposome administration, levels of PDX-1 protein were approximately doubled (Figure 6F).

Although insulin tolerance tests (ITTs) indicated no difference in insulin sensitivity between db/db mice administered empty or clodronate-filled liposomes (Figure 6G), oral glucose tolerance tests (OGTTs) showed that clodronate liposomes ameliorated the glucose intolerance otherwise seen in db/db mice and improved the insulin secretion elicited by oral glucose (Figure 6H). Whereas control db/db mice treated with empty liposomes showed no increase in insulin levels 15 min after oral glucose administration, insulin levels were clearly increased at that time in db/db mice treated with clodronate liposomes (Figure 6H) and were significantly correlated with glucose levels throughout the first 15 min of the OGTT (Figure S7C). This suggests treatment with clodronate liposomes improved insulin secretion in response to glucose. Moreover, when the first-phase insulin response was examined, insulin levels in db/db mice treated with empty liposomes were paradoxically reduced 2 min after intravenous glucose injection, but levels in db/db mice treated with clodronate liposomes were increased at the same time point (Figure S7D). The acute insulin response (AIR)

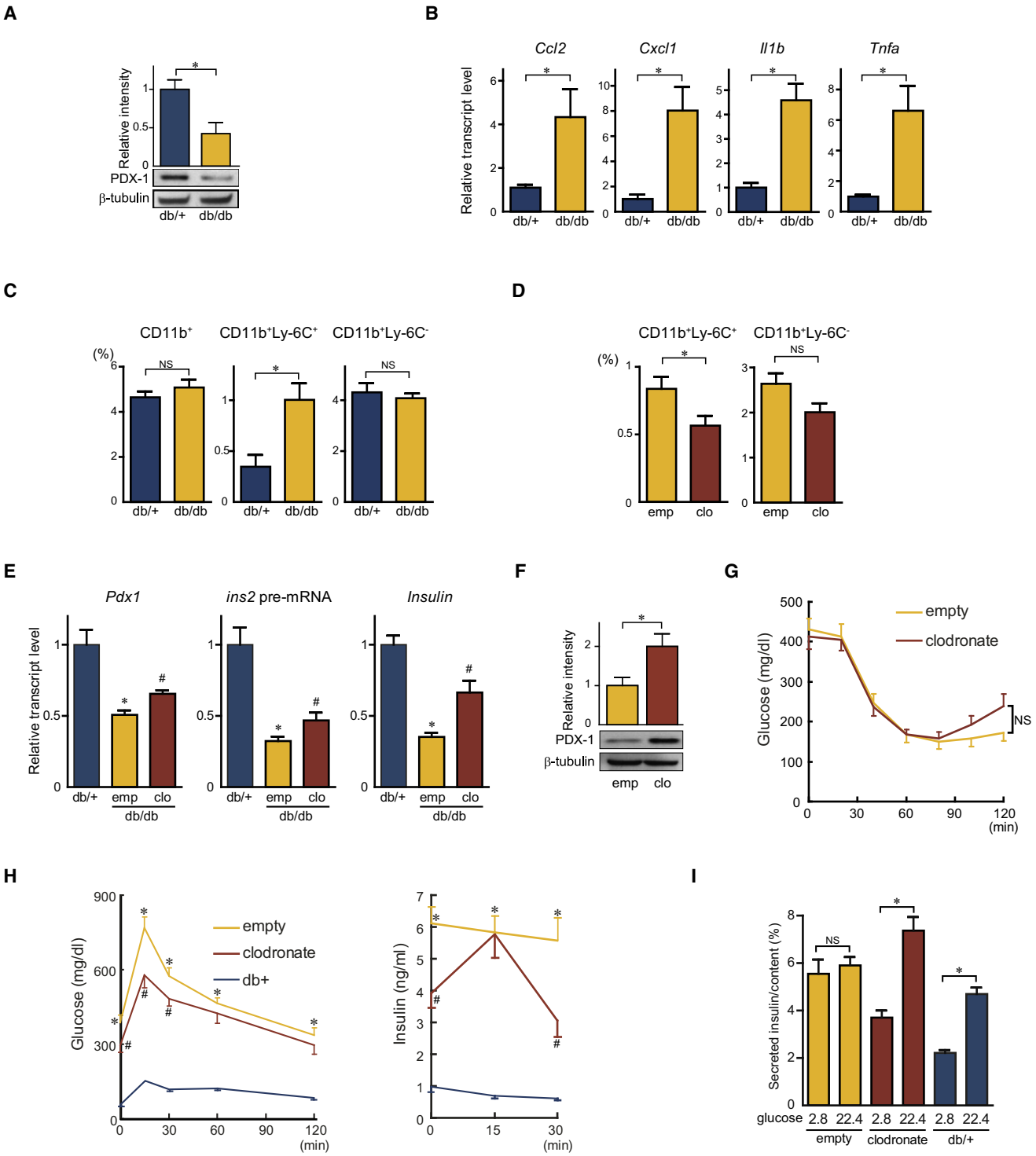

Figure 6. Inflammatory Macrophages Are Crucially Involved in β Cell Dysfunction in db/db Mice

(A) Expression of PDX-1 protein in islets of 10-week-old mice. Relative band intensities were normalized first to those of β-tubulin and then further normalized to the levels in db/+ mice. n = 3 mice for each group.

(B) Expression of β cell-associated genes and proinflammatory genes was analyzed in islets isolated from 8-week-old db/+ and db/db mice. Expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in db/+ mice. n = 4 mice for each group.

(C) Flow cytometric analysis of CD11b$^+$ macrophages within islets. Islets were harvested from 8-week-old db/+ and db/db mice. n = 3 mice for each group.

(D–H) db/db mice were administered clodronate (clo) or empty (emp) liposomes weekly, beginning when they were 5 weeks old. The mice were then analyzed at 10 weeks old, except islet macrophages were analyzed at 8 weeks and ITTs were carried out at 9 weeks. Islet macrophages were analyzed by flow cytometry (D). Empty liposomes: n = 9 mice; clodronate liposomes: n = 6. Expression of β cell-associated genes within islets was analyzed (E). Expression levels were

can become negative in T2D subjects (Mari et al., 2008), and the recovery of the AIR from negative to positive reportedly associates with improvement of β cell function in T2D subjects (Li et al., 2004). Therefore, the recovery of the AIR after clodronate liposome administration suggests there was an amelioration of β cell dysfunction. Furthermore, GSIS was restored in isolated islets from db/db mice administered clodronate liposomes (Figure 6I). Taken together, these results strongly suggest that inflammation plays a key role in β cell dysfunction in db/db mice.

To further confirm that islet inflammation is involved in β cell dysfunction in T2D, we utilized another widely used T2D model, KKAy mice. As was seen in mice infused with ethyl palmitate and in db/db mice, the numbers of $CD11b^+Ly\text{-}6C^+$ cells were increased in the islets of KKAy mice fed a high-fat diet (Figure 7A). Levels of *Insulin* and *Ins2* pre-mRNA expression were significantly lower in islets isolated from KKAy mice administered empty liposomes than in untreated KKTa mice, whereas *Pdx1* levels did not significantly differ (Figure 7B). Clodronate liposome administration increased expression of *Insulin*, *Ins2* pre-mRNA, and *Pdx1* mRNA (Figure 7B), as compared to administration of empty liposomes. OGTTs showed that glucose tolerance was improved in KKAy mice administered clodronate liposomes (Figure 7C), and both OGTTs and IVGTTs showed that there was recovery of the early insulin response to glucose (Figures 7C and S7E). In ITTs, basal glucose levels and levels 20 and 40 min after insulin injection were reduced in KKAy mice administered clodronate liposomes (Figure 7D). In addition, GSIS was restored in islets isolated from KKAy mice administered clodronate liposomes (Figure 7E). These results indicate that clodronate liposomes ameliorated the β cell dysfunction in KKAy mice.

DISCUSSION

The results of the present study demonstrate that (1) the saturated FA palmitate induces β cell dysfunction in vivo; (2) β cells produce chemokines, which recruit $CD11b^+Ly\text{-}6C^+$ M1-type cells to islets in response to palmitate via the TLR4/MyD88 pathway; (3) M1-type cells play a pivotal role in palmitate-induced β cell dysfunction; (4) palmitate induces reciprocal interactions between β cells and macrophages, further augmenting macrophage recruitment and β cell dysfunction; and (5) M1-type cell recruitment and inflammation also play a key role in β cell dysfunction in db/db and KKAy mice. Those results suggest a model in which β cells respond to endogenous stimuli, including saturated FFAs, by initiating macrophage recruitment through production of chemokines, and the subsequent interactions between β cells and M1-type cells propagate and perpetuate inflammatory processes within islets that lead to β cell dysfunction (Figure 7F). Our results thus highlight the causative involvement of inflammation in β cell dysfunction in T2D. They also demonstrate that inflammation is integral to lipotoxicity in vivo.

Our findings demonstrate that β cells play a primary role in sensing palmitate and initiating inflammatory processes within islets. Interestingly, however, *Ccl2* and *Cxcl1* expression was markedly augmented in MIN6 cells cocultured with RAW264.7 cells (Figure 4B), while *Il1b* and *Tnfa* expression was augmented in RAW264.7 cells cocultured with MIN6 cells (Figure 4D). This suggests that once M1 macrophages have accumulated within islets, β cells and macrophages interact reciprocally, at least in part via cytokines, to further augment and propagate inflammatory processes within the islets. In that regard, our findings suggest that blocking IL-1β might improve blood glucose levels and β cell function in T2D patients (Donath and Shoelson, 2011; Larsen et al., 2007) by interfering with the inflammatory interaction between β cells and macrophages. This notion is consistent with the finding that neutralizing IL-1β and TNF-α suppressed the interaction between MIN6 and RAW264.7 cells, but did not ameliorate the direct deleterious effect of palmitate on MIN6 cells cultured alone (Figure S6B).

Earlier immunohistochemical studies showed that macrophages accumulate within the islets of patients with T2D (Ehses et al., 2007; Richardson et al., 2009) and animal models of T2D, including diet-induced obese and db/db mice (Ehses et al., 2007). However, their causative involvement in β cell dysfunction had not been directly tested (Ehses et al., 2008). Our flow cytometric analysis unequivocally indicates that $CD11b^+Ly\text{-}6C^+$ monocytes/macrophages are recruited to islets in mice treated with ethyl palmitate, as well as db/db and KKAy mice, and those recruited cells exhibit the phenotype of M1 activation (Geissmann et al., 2010; Mosser and Edwards, 2008). Clodronate liposomes inhibited accumulation of $CD11b^+Ly\text{-}6C^+$ M1-tpe cells and ameliorated β cell dysfunction in ethyl palmitate-infused, db/db, and KKAy mice, clearly indicating that the recruited M1 cells play a causative role in β cell dysfunction. Previous studies have shown direct deleterious effects of FFAs on β cells; this is collectively termed "lipotoxicity" (Fonseca et al., 2011). In the present study, direct effects of palmitate on β cells were also observed in cultured MIN6 cells (Figure 4B). However, our results indicate that, in addition to the direct toxic effects of lipids, inflammatory processes play a pivotal role in β cell lipotoxicity in vivo. Given the recent findings that M1-type macrophages accumulate in adipose tissue (Hotamisligil, 2006; Maury and Brichard, 2010) and liver (Baffy, 2009) in obese and T2D subjects, the results of the present study suggest the shift in macrophage polarization is broadly involved in the metabolic dysfunction associated with obesity seen in multiple organs. In addition, as FFAs appear to induce cellular dysfunction in all those tissues, our findings also suggest that the activation of inflammatory processes by

normalized first to those of 18 s rRNA and then further normalized to the levels in untreated db/+ mice. db/+: n = 3 mice; db/db administered empty liposomes: n = 9 mice; clodronate liposomes: n = 6. *$p < 0.05$ versus db/+. #$p < 0.05$ versus empty liposome. Error bars, SEM. Levels of PDX-1 protein are shown (F). Relative band intensities were normalized first to those of β-tubulin and then further normalized to the levels in mice administered empty liposomes. Empty liposomes: n = 4 mice; clodronate liposomes: n = 5. Results of ITTs (G) and OGTTs (H). In (G), 2.5 IU/kg insulin were intraperitoneally administered to each mouse; n = 6 or 7 mice for empty and clodronate liposomes, respectively. In (H), 0.5 g/kg glucose were orally administered; n = 16 or 10 mice for empty or clodronate liposomes, respectively. Untreated db/+ mice (n = 8) were similarly analyzed. *$p < 0.05$ versus db/+. #$p < 0.05$ versus empty liposome. Error bars, SEM.
(I) GSIS in islets isolated from db/db mice treated with either empty or clodronate liposomes and db/+ mice. n = 6 islet preparations for each group.

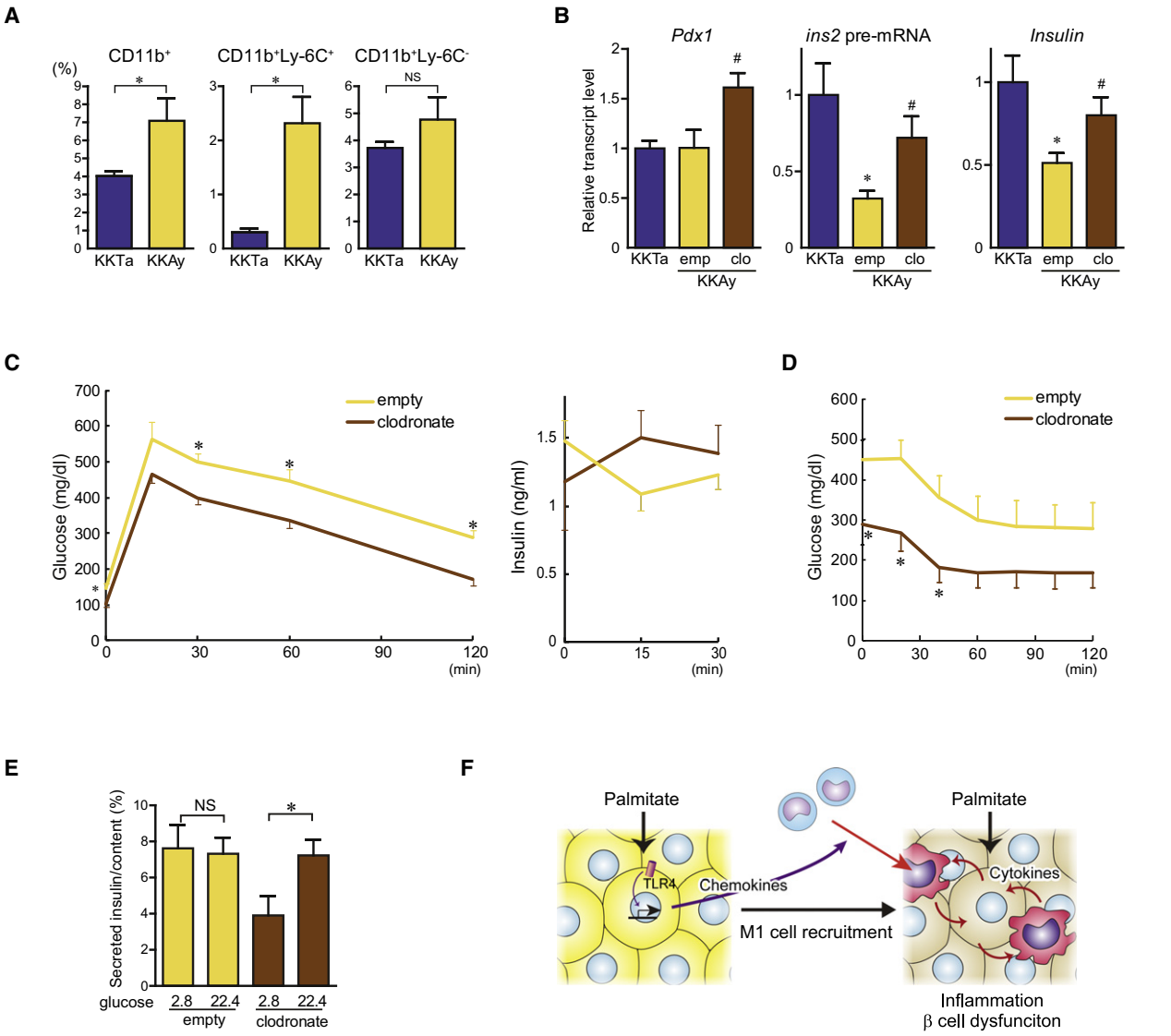

Figure 7. Involvement of Inflammatory Macrophages in β Cell Dysfunction in KKAy Mice

(A) Flow cytometric analysis of CD11b$^+$ macrophages within islets. Islets were harvested from 8-week-old KKTa mice (n = 5) and KKAy mice fed a HFD from the age of 5 weeks. n = 3 mice in each group.

(B–D) KKAy mice fed a HFD from the age of 5 weeks were administered clodronate or empty liposomes weekly, beginning when they were 5 weeks old. The mice were analyzed when they were 7 weeks old, except OGTTs were administered at 6 weeks. Expression of β cell-associated genes within islets was analyzed (B). Expression levels were normalized first to those of 18 s rRNA and then further normalized to the levels in untreated KKTa. KKTa: n = 3 mice; KKAy administered with empty liposomes: n = 6; clodronate liposomes: n = 4. *$p < 0.05$ versus KKTa. $^{\#}p < 0.05$ versus empty liposome. Error bars, SEM. In (C), glucose and insulin levels during OGTTs entailing oral administration of 1 g/kg glucose are shown; n = 6 and 8 mice for empty and clodronate liposomes, respectively. In (D), 2 IU/kg insulin were administered; n = 7 and 9 mice for empty and clodronate liposomes, respectively. *$p < 0.05$ versus empty liposome. Error bars, SEM.

(E) GSIS in islets isolated from db/db mice treated with either empty or clodronate liposomes. n = 6 islet preparations for each group.

(F) A model of palmitate-TLR4 pathway lading to islet inflammation and β cell dysfunction.

FFAs may be part of a unified pathogenic mechanism of metabolic disease.

Previous studies showed that ER stress and ROS pathways are important for β cell lipotoxicity in vitro (Cnop, 2008; Fonseca et al., 2011). We found that ER stress markers were induced by palmitate (K.E. and I.M., unpublished data) as previously reported. Our finding that TLR4 was dispensable for the palmitate-induced downregulation of β cell-associated genes in MIN6 cells makes it likely that ER stress and ROS signaling can be activated independently of TLR4 signaling. Consistent with that idea, we found that knocking down *Tlr4* did not affect induction of ER stress markers in MIN6 cells (K.E. and I.M., unpublished data). Although our results obtained with the ethyl palmitate infusion model and db/db and KKAy mice clearly demonstrate the key involvement of inflammation in lipotoxicity in vivo, cell autonomous mechanisms mediated by those pathways also very likely contribute to β cell lipotoxicity, as reported previously (Hotamisligil, 2010).

In the present study, we surmised that selective elevation of palmitate levels among the other circulating FFA species would be the key to our ability to clearly demonstrate activation of inflammation via the TLR4 pathway. This is because each FA species likely affects metabolism and inflammation differently. For instance, n-3 PUFAs such as eicosapentaenoic acid are known to exert anti-inflammatory effects (Calder, 2009). It is therefore tempting to speculate that the effects of proinflammatory FFA species, such as palmitate, are dampened by anti-inflammatory FFA species, such as n-3 PUFA, in the circulation. However, the shift in balance between proinflammatory and anti-inflammatory FAs due to, for example, consumption of a diet rich in saturated FAs may induce low-level inflammation via the mechanism demonstrated in the present study, which can eventually lead to β cell dysfunction and T2D. This model is supported by human studies showing an association between dietary and plasma FA compositions with low-grade inflammation (Giugliano et al., 2006; Klein-Platat et al., 2005).

In conclusion, the results of the present study clearly demonstrate that activation of inflammatory processes within islets leads to β cell dysfunction. The results also strongly suggest that inflammation is crucially involved in β cell dysfunction in T2D. The molecular and cellular interactions identified in the present study could thus provide novel therapeutic targets for the treatment of T2D.

EXPERIMENTAL PROCEDURES

Ethyl Palmitate Infusion

Ethyl palmitate (Tokyo Chemical Industry) was dissolved with 1.6% lecithin (Wako) and 3.3% glycerol in water to produce the mixture of 600 mM ethyl palmitate, 1.2% lecithin, and 2.5% glycerol. This mixture was then emulsified using a sonicator. The lecithin-glycerol-water solution was used as the vehicle. Endotoxin levels were less than the detection limit of the assay kit (0.015 EU/ml) (Limulus ES-II, Wako). The right jugular veins of two-month-old C57BL/6 mice (average body weight: 23 g) were catheterized using Alzet indwelling catheters. Following a 100 μl bolus injection of either emulsified ethyl palmitate solution or vehicle, the mice were continuously infused with the solutions at 0.2 μl/min.

Serum FFA Measurement

Serum lipids were extracted and separated by thin-layer chromatography (TLC) using hexane/diethyl ether/acetic acid (80:20:1, v/v). FFA spots were revealed using iodine vapor, scraped, and methylated using 1% H_2SO_4 in methanol. Methylated FAs were then quantified by GS-MS analysis.

Clodronate Liposomes

To deplete circulating monocytes, mice were intraperitoneally injected with 0.01 ml/g body wt clodronate liposomes. For the ethyl palmitate infusion experiments, clodronate liposomes were administered 54 and then 6 hr prior to the ethyl palmitate infusion. With db/db and KKAy mice, clodronate liposomes were administered weekly, beginning when the mice were 5 weeks old.

Immunohistochemical Staining

For CD11b staining of whole-mount pancreas, small pieces of pancreatic tissues were rinsed, fixed in acetone, blocked with 2% bovine serum albumin in PBS-T, and immunostained. The stained samples were observed using a confocal laser-scanning microscope (LSM510 Meta, Zeiss). For F4/80 staining, formalin-fixed paraffin-embedded mouse pancreatic sections were labeled with an anti-F4/80 primary antibody (serotec; clone CI:A3-1, 1:400), which was visualized through HRP-DAB detection.

Cell Culture

The murine MIN6 β cell line was kindly provided by Jun-ichi Miyazaki (University of Osaka).

Cell Migration Assay

Migration assays were performed using Boyden chambers. RAW264.7 cells were plated on HTS FluoroBlok inserts with 3 μm pores (Falcon) in serum-free DMEM. Media conditioned with MIN6 cells transfected with siRNA were diluted 10 times and added to the lower wells of Boyden chambers.

Statistical Analysis

Data are shown as means ± SEM. Differences in body weight changes were analyzed using two-way ANOVA. Differences between two groups were analyzed using Student's t test. Comparisons among multiple groups were made using one-way ANOVA followed by a post hoc Tukey-Kramer test for multiple groups. Values of $p < 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2012.01.023.

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